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Detection of Pan Braf in Thyroid Tumors in Iraqi Patients

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Abstract

The B-type Raf kinase (BRAF) is a member of RAS\RAF\MEK\ERK pathway and this pathway can lead to increased cellular growth, invasion and metastasis. The mutated BRAF protein activates MAPK signaling pathway, results in abnormal cellular growth, apoptosis resistance, tumor progression and metastasis. Pan-BRAF is one of available BRAF monoclonal antibodies and shared by both the wild and mutant BRAF.BRAF status is mostly determined by DNA sequencing methods. In this investigation we assessed the monoclonal Pan BRAF specific antibody that can identify wild and mutant type proteins together in formalin-fixed paraffin-embedded thyroid tumor tissues by Immunohistochemistry (IHC). Archival thyroid samples from 43 iraqi patients were immunohistochemically tested with antibodies for BRAF. Out of 43 thyroid tissue cases, (23) were thyroid malignant,(12) benign, and (8) control cases(diagnosed as colloid goiter).The malignant tumors included Papillary Thyroid Carcinoma (PTC), Follicular Thyroid Carcinoma (FTC), Medullary Thyroid Carcinoma (MTC), Anaplastic Thyroid Carcinoma (ATC) and Hürthle cell cancer (HCC).Immunohistochemical staining for BRAF was performed for all specimens. Results of the study showed that Immunohistochemical expression of pan BRAF was significantly higher in malignant thyroid tumors as compared with adenomas and control cases ($P<0.05$). BRAF over-expression was detected in 5\12 of PTC, 3\5 MTC, 2\4 of FTCas well as all cases of HCC, ATC.Whereas it was detected in 4\12 of adenomas, and totally negative in control cases. No association was observed between BRAF and other clinicopathological traits. We conclude from this study that IHC using BRAF monoclonal antibody is a successful way for checking of BRAF status in different thyroid tumors. IHC may be the alternative to molecular biology for the routine detection of this marker in patients with thyroid tumors.

Keywords: BRAF Monoclonal antibodies, Immunohistochemistry, Thyroid Carcinoma.

التحري عن Pan BRAF في اورام الغدة الدرقية في المرضى العراقيين

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الخلاصه

يعد Raf kinase نوع B عضوا" في مسار *RAS\RAF\MEK\ERK* ، وهذا المسار يمكن أن يؤدي إلى زيادة النمو الخلوي cellular growthوالاختراق invasion والانبثاثmetastasis. ان بروتين BRAFالمطفّر ينشط مسار MAPK،مايؤدي الى نمو خلوي غير طبيعي، ومقاومة عملية موت الخلايا المبرمج، وتطور الورم والانبثاث. يعدPan BRAF واحدا من اشكال الأجسام المضادة وحيدة النسيلةالمتاحة

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والتي تظهر في كل من BRAFالعادي والمطفر. يتم تحديد حالة BRAFغالبا عن طريق تسلسل الحمض النووي. في هذه الدراسة تم تقييم الأجسام المضادة وحيدة النسيلة ل *Pan BRAF* التي يمكنها تشخيص بروتين BRAFالعادي والمطفر معا في الأنسجة الماخوذة من اورام الغدة الدرقية بطريقة الكيمياء النسجية المناعية*Immunohistochemistry (IHC)* . تم فحص عينات الغدة الدرقية المحفوظة بالبرافين والماخوذة من 43 مريضا عراقيا بطريقة الكيمياء النسجية المناعية باستخدام الأجسام المضادة ل BRAF. من أصل 43 حالة، كان (23) منها مصابة باورام الغدة الدرقية الخبيثة، (12)حالة ورم حميد، و (8) حالات السيطرة (شُخصت على انها تضخم الغدة الدرقية الغرواني). شملت الأورام الخبيثة سرطان الغدة الدرقية الحليمي (PTC)، وسرطان الغدة الدرقية الجريبي (FTC)، وسرطان الغدة الدرقية النخاعي (MTC)، وسرطان الغدة الدرقية المتحول (ATC) وسرطان الخلايا هورثل (HCC). تم إجراء تصبيغ الكيمائي المناعي لجميع العينات. اظهرت نتائج الدراسة ان التعبير التعبير الكيميائي المناعي ل *pan BRAF*أعلى بكثير في أورام الغدة الدرقية الخبيثة بالمقارنة مع الأورام الحميدة وحالات السيطرة (*P<0.05*). تم ملاحظة فرط التعبير ل*BRAF* في 5 \ 12 من حالات PTC، 3 \ 5 من حالات MTC، 2 \ 4 من اورام ال FTC وكذلك جميع حالات HCCو ATC. في حين تم الكشف عنها في 4 \ 12 من الاورام الحميدة، فيما كانت النتائج سلبية تماما في حالات السيطرة.كمالم يلاحظ أي ارتباط بين BRAF والصفات السريرية والمرضية. نستنتج من هذه الدراسة ان طريقةالكيمياء النسجية المناعية باستخدام الاجسام المضادة وحيدة النسيلة لBRAF هي وسيلة ناجحة للتحقق من حالة BRAF في أورام الغدة الدرقية المختلفة. قد تكون طريقةالكيمياء النسجية المناعيةالبديل للبيولوجي الجزيئي للكشف الروتيني لهذا المؤشر في المرضى الذين يعانون من أورام الغدة الدرقية.

I. Introduction

Thyroid cancer (TC), the major endocrine tumor, has been increasing rapidly since the last 30 years[1].The rising number of low-stage TC;Papillary Thyroid Carcinoma (PTC)increased the controversy about the best therapeutic strategy [2]. The diagnosis of thyroid cancer is well in general. However, there are up to 15% of patients develop local or distant recurrences [3]. Searching for molecular markers is a promising way to create strategies for suitable patients' categorization to avoid the risk of ineffective treatment among high-risk patients [4]BRAF, the most common oncogene that observed in about 50% of PTC, is one of the best candidates [5].This marker is generally negative in benign follicular lesions, normal thyroid tissue, medullary thyroid carcinoma (MTC), and follicular thyroid carcinoma (FTC)[6].Currently, the detection of this marker is of strong interest in medical routine and is well established [7].

A lot of attention has been paid to the prognostic and therapeutic prospective of BRAF. Overexpression of this protein in TC causes constitutive activation of oncogenic pathways critical to PTC tumorigenesis[8].Pan-BRAF is one of available BRAF monoclonal antibodies and shared by both the wild and mutant BRAF. Most malignancies display diffuse pan-BRAF staining (BRAF over-expression) despite of BRAF mutation status [9]. The prognostic significance of BRAF over-expression has been analyzed widely, with controversial conclusions [10, 11]. Many studies showed an association of the BRAF overexpression with aggressive features of TC [12].

Evaluation of BRAF has been recommended by several recent studies since this step can assist in the surgical and/or medical managing of patients with thyroid carcinoma [13]. A recent retrospective study concluded that patients with BRAF-positive tumors are at increased risk for cancer-related mortality [14].Other meta-analysis study recommended that papillary thyroid carcinoma (PTC) with the BRAF mutation is related to higher risk of recurrent, lymph node metastasis, and extra thyroidal extension [7].

BRAF status is mainly assessed by DNA-based methods, most commonly by sequencing. However, such methods tend to be costly, prolonged, and difficult to be confirmed and applied in some clinical settings [15].Lately, immunohistochemistry(IHC) using BRAF specific antibody has been used for detection the alterations of this protein in several types of malignancies, including TC [6, 16]. BRAF monoclonal antibody has been proven to be useful in detection of BRAF status, with a sensitivity and specificity of more than 95% when compared to other molecular methods[17].Actually, some studies recommended that BRAF specific antibody is more sensitive than molecular testing in detecting the BRAF mutation[18, 19].The objective of our study was to evaluate the efficacy of

immunohistochemistry(IHC) in detection the over-expression of BRAF(pan-BRAF)in paraffin-embedded thyroid tumor tissues using monoclonal BRAF specific antibody.

II. Materials and Methods

Archival thyroid samples from histopathology unit\ central public health in Baghdad were retrospectively analyzed. These cases included 23 malignant, 12 benign, and 8 cases as control.H & E stained sections were re-assessed by a pathologist. Pan BRAF proteins were detected by Immunohistochemistry using Rabbit anti pan BRAF monoclonal antibody. Patients' consent was taken.

Immunohistochemistry

Immunohistochemical staining procedure was carried out according to general protocol of immunohistochemistry. Sections were dewaxed in xylene and rehydrated in ethanol. Antigen was retrieved using Tri-sodium citrate buffer (pH 6.0 to 6.2) plus microwave oven. Next, slides were incubated in peroxidase – blocking solution (Dako, ready- to- use) for 20 min. Non-specific binding of antibodies was blocked by 2.5% of normal horse serum, from (ImmPRESS, Vector, USA). Then, primary antibody for BRAF (Rabbit monoclonal antibody,clone EP152Y[ab33899],ABCAM, Cambridge, UK) was diluted(1:250)using antibody diluant (ready-to-use, Code No. [ab64211] ABCAM, Cambridge, UK), and incubated for 1hour at room temperature.After that, secondary antibodies (Anti Rabbit Ig. peroxidase, Cat. No. MP-7401, ImmPRESS™ Vector, USA) was applied to the slides and incubated for 30 minutes at room temperature in a humidified chamber. The colorimetric reaction was detected by the diaminobenzidine (DAB) Peroxidase Substrate method. After that, sections were counterstained with haematoxylin, dehydrated, and mounted.

BRAF Immunohistochemical Scoring

Scoring for all the immunohistochemical expression results were assessed by a specialist pathologist. The scoring of BRAF was done semi quantitatively according to Fisher *et al.* (2014), depending on the observing of a diffuse dark cytoplasmic staining in neoplastic cells.Cut of value is **>10%** of moderate and strong intensity of cytoplasmic tumor cells are considered positive expression of BRAF, while **< 10** of any intensity as well weak intensity in **>10**of tumor cells is considered negative expression.

Statistical analysis

All cases were analyzed using SPSS 20. Chi-square test was used to calculate P value. P value of <0.05 was considered as significant.

III. Results

The mean age of studied cases was (36.9±11.17) years; ages ranged from(20-75)years. Results showed no significant correlation between expression of BRAF and age in malignant cases (P=0.3) Figure-1.

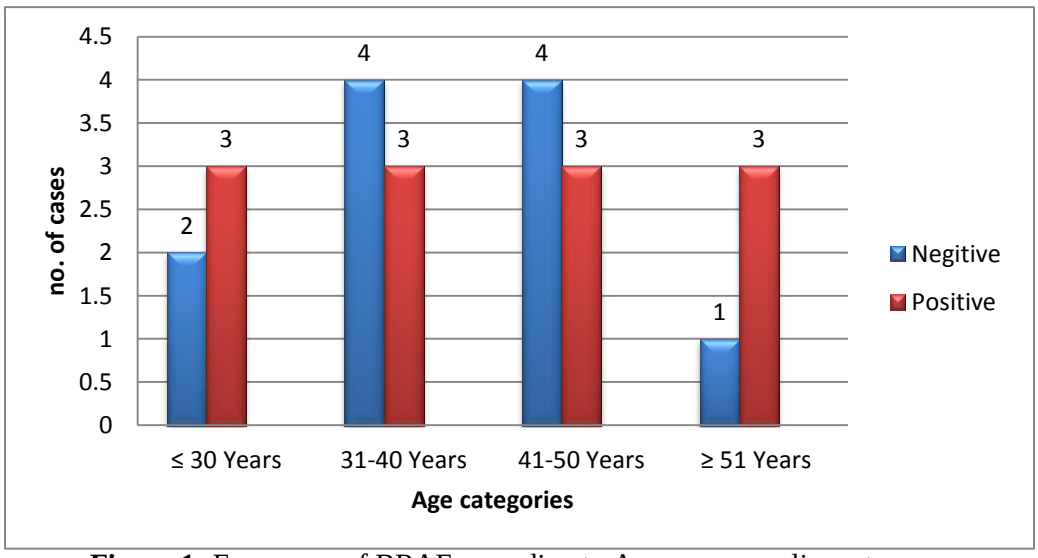


Figure 1- Frequency of BRAF according to Age among malignant cases.

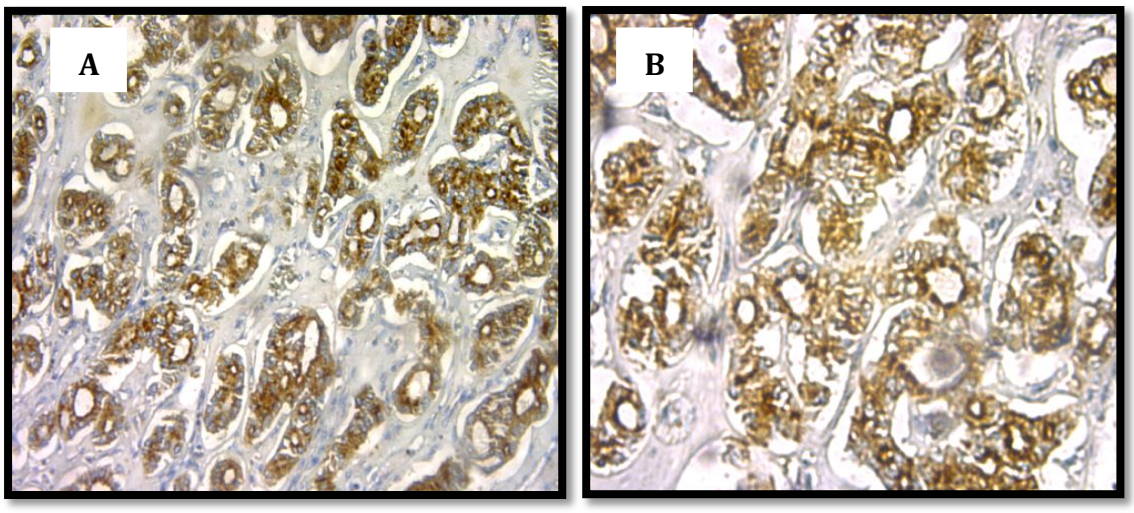
The malignant cases constituted 23 (64%) of total, distributed as 12PTC, 5MTC, 4FTC, 1ATC and1HCC. While benign cases comprised 12 (28%).And the residual 8cases (19%) were used as control. BRAF was positively expressed in 12\23 (52%) of malignant cases and in 4\12 (33%) of adenomas. Alterations of BRAF were noticed in 5\12 (42%) of PTC, 2\4 (50%) of FTC, 3\5 (60%) of MTC, as well as all cases (100%) of HCC and ATC, in comparison with control cases which showed totally negative expression (100%) when stained with this marker Table-1, Figure-2. Details of intensity scoring were illustrated in Table-2. No significant correlations were recorded in the expression of BRAF among histological types.

Table 1-Immunohistochemical expression of BRAF according to histological types.

	Histological Types						
Score	**Normal**	**Adenoma**	**PTC**	**FTC**	**MTC**	**HCC**	**ATC**
Negative	**8(100%)**	**8(67%)**	**7(58%)**	**2(50%)**	**2(40%)**	**0**	**0**
Positive	**0**	**4(33%)**	**5(42%)**	**2(50%)**	**3(60%)**	**1(100%)**	**1(100%)**
Total	**8**	**12**	**12**	**4**	**5**	**1**	**1**

Table 2-Immunohistochemical intensity of BRAF in different histological types of thyroid tissues

Histological types	**No. of cases**	**No Stain /0+ Negative n %**	**Weak/1+**		**Moderate/2+**		**Strong/3+**		**Total positive %**	**Total Negative %**
			<10	**>10**	**< 10**	**> 10**	**< 0**	**>10**		
Malignant	**23**	**3 (13%)**	**5**	**2**	**1**	**5**	**0**	**7**	**12(52%)**	**11(48%)**
Benign	**12**	**2 (17%)**	**4**	**1**	**1**	**4**	**0**	**0**	**4(33%)**	**8(67%)**
Control	**8**	**8 (100%)**	**0**	**0**	**0**	**0**	**0**	**0**	**0/(0)**	**8(100%)**
Total (+/-)	**43**	**13/-**	**9/-**	**3/-**	**2/-**	**9/+**	**0/-**	**7/+**	**16(37%)**	**27(63%)**



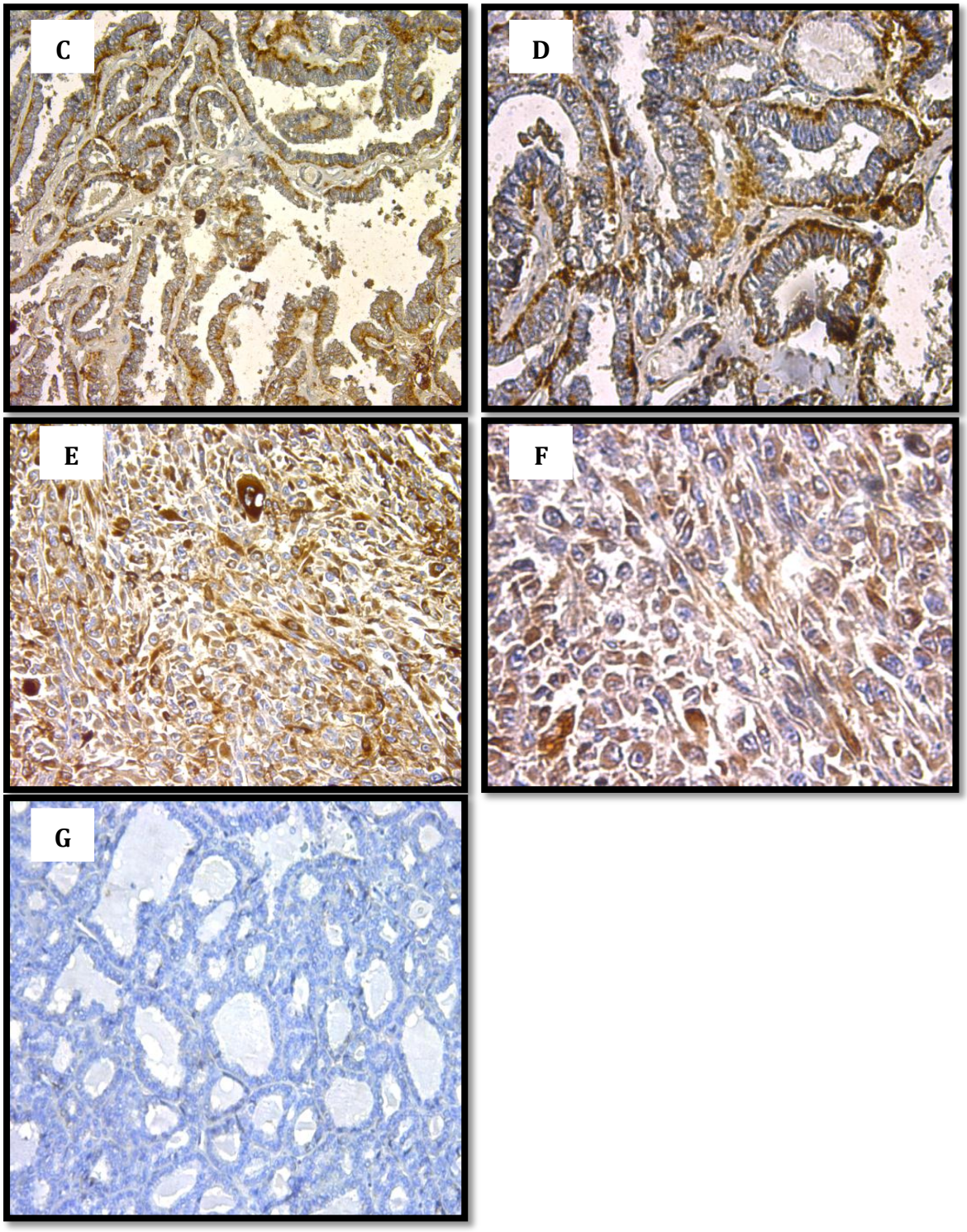


Figure 2- Sections of different types of thyroid carcinoma in different magnification powers: (A, B) showed PTC; (C,D) showed FTC; (E,F) showed ATC and (G) showed negative control. [Uniform positive reaction for BRAF in tumor cells, strong cytoplasmic reactivity in > 10% of tumor cells, (brown stain for cytoplasmic of carcinoma cells), A, C, E, and G X20; B, D, and F X40. PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; ATC, Anaplastic thyroid carcinoma]

Also in this study, the majority of tumor cases were women (26\35; 61%).No significant associations were detected in the expression of BRAF between men and women in this study (P=0.5) Table-3.

Table 3- Association of BRAF expression with sex

IHC expression of BRAF			***P* value**
Sex	**Negative**	**Positive**	**0.5**
Male	**10(59%)**	**7(41%)**	
Female	**13(50%)**	**13(50%)**	
Total	**23/43**	**20/43**	

The present study revealed that BRAF alterations were significantly higher in malignant thyroid tumors when compared with adenoma and control cases (P=0.03)Table-4

Table 4-Association of BRAF expression with histological types

Histological types				***P* value**
BRAF expression	**control**	**Adenoma**	**Malignant**	**0.03***
Negative	**8(100%)**	**8(67%)**	**11(48%)**	
Positive	**0**	**4(33%)**	**12(52%)**	
Total	**8**	**12**	**23**	

***P<0.05**

IV. Discussion

Right now, there are inconsistencies in the viewpoints about the role of BRAF as a prognostic factor inTC[20].The predictive importance of this marker has been controversial for 10 years[21].The evaluation of BRAF protein expression is of clinical importance, particularly in therapy-resistant disease, as some new medicines inhibiting the transformed protein is clinically offered [22]. In this study, we examined the efficacy of pan BRAF monoclonal antibody that identifies both wild and mutant BRAF protein. This developed antibody recognizes the N-terminal end on both sides of amino acids (70 – 86)that is displayed by both the wild and mutant BRAF protein [8]. Up to our knowledge, this is the first study in Iraq to assess the over-expression of BRAF by IHC in TC.

Our study revealed that BRAF alterations were observed in (52%) of malignant cases; it was detected in (42%) of PTC, (50%) of FTC, (60%) of MTC as well as all cases of HCC and ATC, without significant association with neither age nor gender. This insignificant correlation with sex could belong to the low number of male's malignant cases of this study.Our results have the same opinion with a prior study showed that BRAF was overexpressed in 60% of PTC as well as 74% of FTC and the results associated significantly with patients' <45yrs, but without significant differences between men and women (22). Other finding byFisher et al., who declared that Pan-BRAFwas observed in 80.5% of cases: 34.1% were with BRAF mutations and 46.3% were wild type[8].Whereas current outcome partially agree with what were obtained by several previous studies, of them; Jong, *et al.*,who recorded that *BRAF* expression was detected in 68 % (71/104) of PTC butit was not detected in patients with FTC(0/18) or in MTC(0/21)[21]. In addition to other study which detected BRAF in

54.5% of TC cases and found that patients withBRAF mutation were older (>45) than patients lacking the mutation (p<0.01), without significant differences between men and women[2]. Furthermore, a study by Koperek, *et al.*,found that 76\144 (52.8%) of tumors showed cytoplasmic expression of BRAF protein, and BRAF protein expression significantly correlated with patients'age(P=0.007)as well as tumor size(P=0.018), but not with gender(NS)[23].Also, Zhua et al., 2016 detectedBRAF in 68.6% (81/118) of PTC samples by IHC and reported that IHC has high practical value for the detection of the BRAF V600E mutation in metastatic and primary PTC[24].

Moreover in this assay, BRAF was detected in 33% of adenomas.Similar to our outcome, Sapio *et al*. who detected no mutations of BRAF in the normal thyroid tissues as well as follicular adenomas[25] as well as Atik*et al.*,who found no association between follicular adenoma and BRAF gene mutation (*P* >0.05) but its detection can be a useful tool combined with immunohistochemistry for diagnosing FTC [26].

The variations in BRAF expression among the published findings may be due to either the differences in size of studied samples[27] or to loco-regional differences in the pathogenesis of TC[28] or can due to different analyzing methods used for the detection of the protein[29, 30]. Our data show that the antibody directed against the BRAF protein reliably identifies TC harboring the BRAF alterations. This finding come with harmony to our previous study including 47 of breast cancer tissue samples that could be successfully detected BRAF overexpression as negative and positive by means of IHC[31], indicating that this method may considerably assist investigation of the BRAF status in TC.

Other hand, our investigation showed that BRAF protein existsin PTC cases with no significant differences among histological types.This agrees with an earlier study; find that BRAF protein occurs early in carcinogenesis of PTC with no significant difference between microcarcinomas and macrocarcinomas[23]. As well, similar results were previously shown by sequencing analysis viewing BRAF alteration with a range (20-52%) in PTCwhich generally has an inactive clinical course [32,33]. Our study showed that the immunohistochemical detection of BRAF can considered as adependable, precise method for detection the alterations of this protein in TC.It provides a potentially rapid, easily applicable, and economic alternative to current techniques [16]. The expression of BRAF protein might be of clinical interest,especially in therapy-resistant disease, as new therapeutics inhibiting the mutated BRAF protein are clinically available[34]. More studies with larger series using more specific antibodies directed for specific BRAF mutations and IHC are needed to improve our understanding of this interesting marker.

Conclusion

Assessment of Pan BRAF monoclonal antibody using IHC technique is a successful way for checking of the BRAF status in different thyroid tumors. IHC may be the alternative to molecular biology for the routine detection of this marker in patients with thyroid tumors.

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Conflict of interest: None

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